2013

Sec14-like proteins, PATELLIN1 and PATELLIN2, are essential for procambial pattern establishment in Arabidopsis thaliana

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Sec14-like proteins, PATELLIN1 and PATELLIN2, are essential for procambial pattern establishment in *Arabidopsis thaliana*

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Submitted in partial fulfillment of the prerequisite for Bachelor of Arts degree with Honors in Biological Sciences

Wellesley College
April 2013

ACKNOWLEDGEMENTS

To Kaye Peterman, thank you for all your food recommendations and for laughing at my bad jokes. But also, thank you for believing in me, pushing me, and being patient with me. Your attentiveness and care became the cornerstone of my Wellesley experience and I will keep those memories close throughout my future adventures.

Thank you to my thesis committee—Yui Suzuki, Gary Harris, and Robert Berg—for your feedback, support, and ideas.

To the Peterman lab past and present, thank you for all the memories and hot summers spent together. Especially, thanks to Angela Ai for watering my plants on more occasions that I can count.

Thank you to my friends and teachers, who make the Wellesley community great.

And to my parents, for encouraging my curiosity and for supporting me.

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ABSTRACT

The directional flow of auxin is canalized during development into continuous files of cells that predict the final vascular pattern. As auxin is polarized within the cell, ground cells acquire procambial cell fate, multipotent stem cells that will differentiate into all the cells of the vascular tissue. Cellular events that lead to procambial state acquisition are not well elucidated. The *PATELLIN1* (*PATL1*) and *PATELLIN2* (*PATL2*) genes, which code for phosphoinositide-binding Sec14-like proteins in *Arabidopsis thaliana,* are expressed in vascular tissue and microarray expression data implicate their involvement in xylem differentiation. We show here that *PATL1* and *PATL2* are necessary for complete vascular pattern development in cotyledons. *patl1 patl2-1* T-DNA insertion mutants exhibited an increased number of gaps, vein order reversals, distal defects, and free ends. Because vascular patterning defects are associated with improper procambial state acquisition, we investigated the expression of *PATL1* and *PATL2* in developing embryo and leaf primordia. *PATL1* and *PATL2* were expressed in preprocambial cells of the developing embryo, leaf primordia, and in mature vascular cells of the cotyledon, suggesting their involvement in procambial state establishment. Furthermore, the DR5::GFP auxin reporter pattern was disrupted in *patl1 patl2-1* mutants which supports the hypothesis that PATL1 and PATL2 regulate polar auxin transport, perhaps by influencing the localization of PIN1. We propose that *PATL1* and *PATL2* function along with ARF-GAPs necessary for polar auxin transport, suggesting a novel role for Sec14-like proteins in *A. thaliana.*

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LIST OF ABBREVIATIONS

AHP6	Histidine phosphotransfer protein 6
ARF	ADP-ribosylation factor
AtHB8	*Arabidopsis thaliana* Homeobox gene 8
AXR6	Auxin resistant 6
BDL	Bodenlos
BP	T-DNA border primer
COW1	Can of Worms 1
CVL1	CVP2-like 1
CVP2	Cotyledon vascular pattern 2
DIC	Differential interference contrast
DR5	Direct repeat 5
GAP	GTPase activating proteins
GEF	Guanine nucleotide exchange factors
GFP	Green fluorescent protein
GNOM	Embryo defective 30
GSP	Gene-specific primer
GUS	β-glucuronidase
IAA	Indole acetic acid
KNOX	Class-I KNOTTED 1-like Homeobox
MP	Monopteros
PATL1	Patellin 1

PATL2	Patellin 2
PI	Phosphoinositide
PIN1	PIN-FORMED1
PtdIns	Phosphatidylinositol
SAM	Shoot apical meristem
SEC14	Secretory 14
siRNA	Short-interfering RNA
smRNA	Small RNA
T-DNA	Transfer DNA
TF	Transcription factor
TGN	Trans-Golgi Network
VAB	V-ATPase B Subunit 3
VAN3	Vascular network defective 3

“But seeds are invisible. They sleep deep in the heart of the earth's darkness, until someone among them is seized with the desire to awaken. Then this little seed will stretch itself and begin—timidly at first—to push a charming little sprig inoffensively upward toward the sun.”

-The Little Prince, Antoine de Saint-Exupéry

INTRODUCTION

The plant vascular system, composed of a continuous network of bundled conduits, accomplishes an incredible feat of carrying water and nutrients, sometimes for hundreds of meters. The complex pattern of this network of veins has fascinated many, among them mathematicians, poets, and biologists. Not only beautiful, the plant vascular system is a developmental marvel—from the point of fertilization, vascular precursors are established and self-assembly of the network begins. As the plant matures, vascular networks must be mechanically rigid enough to supply the organism with vital water and nutrients, but also developmentally plastic enough to adjust to environmental fluxes. The coordination of this replicable, yet adaptable pattern *de novo* is an intriguing phenomenon for developmental biologists.

I. Structure and Function of Vascular Tissue

A. Plant vascular tissue is composed of two cell types: xylem and phloem

The plant vascular system is composed of two tissue types that are bundled together: xylem, responsible for water and nutrient transport, and phloem, responsible for sugar transport. The vascular bundles form a continuous system that extends throughout the entire plant body.

***Xylem*.** Xylem tissue is composed of tracheary elements (conducting cells), xylem parenchyma, and fibers. The tracheary elements are established by the deposition of a secondary cell wall, accumulation of hydrolytic enzymes, thickening of the secondary cell wall, and eventual lysis of the cell (Fukuda, 1997). Thus, through the process of programmed cell death, hollow tracheary elements develop, aligned end-to-end to transport water and nutrients throughout the plant. The first xylem cells to undergo this feat are protoxylem cells, which originate simultaneously in the developing hypocotyl at the base of the cotyledons and the root of the bent cotyledon stage

embryo (Busse and Evert, 1999). The protoxylem cells are identified by annular or spiral secondary wall thickenings. The biogenesis of these cells appears to be closely linked with the ability to deposit glycine-rich proteins and lignin in the secondary cell wall (Keller et al., 1989). Following the establishment of protoxylem, a transcriptional switch initiates the formation of metaxylem elements (Kubo, 2005). Cytokinin is known to play an inhibitory role in the formation of protoxylem; only when a cytokinin modulator AHP6, a pseudophosphotransfer protein, is present can protoxylem form (Mahonen et al., 2006). When cytokinin levels are higher and when AHP6 is not present, metaxylem cells form, characterized by the more complex patterns in their secondary cell walls.

Xylem parenchyma cells are uniform, isodiametric ground cells that do not undergo programmed cell death (Turner and Sieburth, 2003). Xylem parenchyma cells serve as helper cells for the nearby differentiating xylem conducting elements and are involved in stress responses. Short bursts of hydrogen peroxide, produced endogenously by xylem parenchyma cells, diffuse to nearby differentiating tracheary elements to provide the cells with the necessary materials for polymerization of cinnamyl alcohols used for lignification of xylem vessels (Barcelo, 2005). Xylem parenchyma cells have also been associated in maintaining potassium homeostasis and long distance signaling (Wegner and DeBoer, 1997). These cells are able to transport molecules into the xylem vessel system via amino acid transporters, allowing them to play a role in long-distance signaling (Okumoto et al., 2002). Xylem parenchyma cells are also associated with a defense salt-stress response: they are able to unload excess Na^+ from tracheary elements to increase salt tolerance (Sunarpi et al., 2005); they can also unload peroxidases from xylem and signal secondary wall thickening as part of a defense response (Hilaire et al., 2001).

Phloem. Phloem cells flank the xylem cells within the vascular bundle. While xylem tissue transports water and nutrients throughout the plant, phloem tissue is involved in sugar transport. Phloem is composed of a sieve tube element/ companion cell complex that includes three cell types: companion cells, sieve tube elements, and phloem parenchyma cells. Sieve tube elements are enucleate cells that transport primarily sugar throughout the plant; they are connected end-to-end via sieve plates. Sucrose and certain amino acids travel from the leaf apoplasm to minor veins by proton symporters in the plasma membranes of sieve elements (Lalonde et al., 2003). Generally, two phloem parenchyma cells flank the sieve tube element/ companion cell complex. The phloem parenchyma cells act as communicators between the surrounding tissue and the complex; the frequency of plasmodesmata and cell wall ingrowths is very high between phloem parenchyma and sieve tube elements. Increased contact between these cells suggests possible translocation of photoassimilates from bundle sheath cells and may indicate sugar transport into the phloem parenchyma cells (Haritatos, 1999). In addition to sucrose transport, small RNAs (smRNAs), including short-interfering RNAs (siRNAs) are transported via phloem sieve tube elements to direct plant stress-response and development (reviewed in Kehr and Butz, 2008). Together, xylem and phloem tissue make up the cells of the vascular system and accomplish the incredible feat of transporting nutrients, water, and communication signals throughout the plant organs.

II. Development of the vascular system

A. The vascular pattern is initiated during embryogenesis

Throughout the development of the embryo, the vascular pattern emerges. The plant embryo is polarized immediately after fertilization, and cells in the lower-middle tier of the developing embryo are elongated thus establishing vascular precursors (Cano-Delgado et al., 2010). Uniform

ground cells in the embryo become patterned by the polarized localization of auxin, a phytohormone (Sabatini et al., 1999). Auxin identifies and initiates cells destined to become vascular cells, known as preprocambial cells. Preprocambial cell establishment is regulated by AtHB8, a homeobox gene responsive to auxin (Baima et al., 1995). These preprocambial cells differentiate into the procambium, elongated stem cells that will give rise to all cells of the vascular tissue. The pattern of the procambium directly predicts the pattern of the vascular system of the young seedling (Jurgens et al., 1994, Sieburth et al., 1999). Establishment of the procambium begins during the late globular stage, when isodiametric ground cells elongate along the apical-basal axis. The procambial pattern develops further during the heart stage. A pericycle layer surrounds these defined procambial cells and a hypocotyl region can be observed throughout the mature embryonic stages (Busse and Evert, 1999). Differentiation of the vascular cells is arrested until conditions favorable for germination arise. Following germination, patterned cells of the procambium produce the cells that will differentiate into the xylem and phloem (Jurgens et al., 1994).

B. Maturation of the vascular tissue occurs post-embryonically in root, hypocotyl, and stem

After germination, the pattern defined by the procambium differentiates into the cambium—cells that will give rise to xylem and phloem tissue. The maturation of the vascular bundles follows a defined pattern along the apical-basal axis as well as radially in the root, hypocotyl, and the stems. Generally, long files of xylem and phloem extend through the length of the apical-basal axis, carrying important nutrients, sugar, and water. The organization of xylem and phloem within bundles differs in the root, hypocotyl, and stem (Fig. 1).

Root. A diarch pattern of vascular bundles composes the developing root at the root apical meristem (Fig. 1). Major phytohormones have been associated with the vascular patterning

process in the root. Alternating high and low indole acetic acid (IAA) concentrations induce the radial pattern of the protoxylem and protophloem, whereas the differentiation of xylem is induced by a combination of auxin and ethylene (Cano-Delgado et al., 2010). The xylem and phloem cells differentiate shootward, beginning close to the quiescent center within the root giving rise to a vascular cylinder, composed of files of phloem and xylem (Mahonen et al., 2000). As the plant matures and begins to develop lateral roots, the vascular pattern is extended to the newly forming tissue. Cytokinin maintains the continuity of the primary vein during this developmental process (Aloni et al., 2006). Additionally the pericycle cells that surround each vascular bundle is involved in maintaining vascular architecture in the root, especially during the development of lateral roots. *In silico* analysis of pericycle gene expression analysis suggests that some transcription factors (TFs) in the pericycle may be associated with xylem cell fate acquisition within the root, intimately implicating the pericycle in root vascular patterning (Parizot et al., 2012). As such, phytohormones and transcription regulators work to form and maintain the emerging vascular bundle pattern in the root.

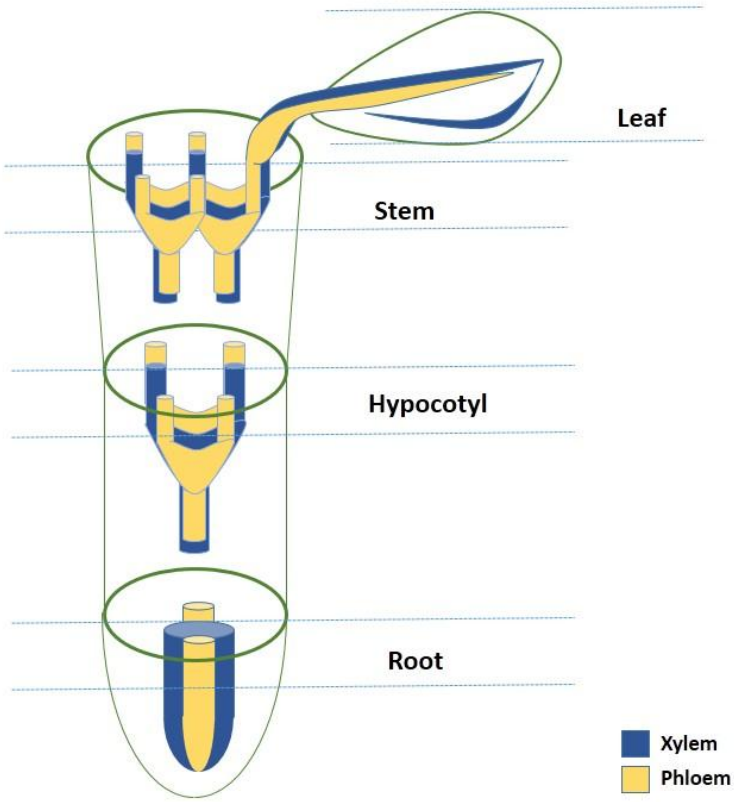


Figure 1. Post-embryonic vascular pattern. The vascular system is composed of long strands of xylem and phloem tissue connecting the entire body of the plant along the apical-basal axis. However, the radial organization of the vascular bundles is different in the root, hypocotyl, and stem.

Hypocotyl. Similarly to the root, the hypocotyl is formed from procambial cells established during embryogenesis. In *A. thaliana* the vascular cylinder within the hypocotyl forms a diarch pattern, composed of xylem and phloem bundles that are separated by yet undifferentiated procambial cells (Fig. 1). The protophloem develops on opposite sides of the vascular cylinder, closest to the pericycle cells, whereas the xylem develops centripetally within the vascular cylinder (Reviewed in Turner and Sieburth, 2003). At the hypocotyl, the vascular bundles become branched and extend into the stem (Fig. 1).

Shoot. The shoot vascular system provides nutrient and water transport along the apical-basal axis and connects the veins of distal organs. At the apex of the hypocotyl or the transition zone, differentiation of leaf vascular tissue begins (Fig. 1). Roughly five to eight vascular bundles are arranged in a ring and interfasicular cells are found in between. The protoxylem differentiates closest to the center of the stem and proceeds distally, forming an endarch pattern (Busse and Evert, 1999). The radial pattern of vascular bundles is tightly controlled both by brassinosteroids, which regulate the number of vascular bundles, and auxin, which regulates vascular bundle spacing in *A. thaliana* shoots (Ibañes et al., 2009).

Precise vascular patterns are observed not only as the plant extends along the apical-basal axis, but also as the plant grows radially. While primary growth is associated with elongation along the apical-basal axis, secondary growth in plant tissues is driven by meristematic activity in the vascular cambium and is associated with expansion around the center (Sehr et al., 2010). In *Arabidopsis* and other dicots, secondary growth occurs in the hypocotyl and root apical meristem. Roughly six days post germination, a secondary growth of the hypocotyl is initiated, first noted by the maturation of xylem vessel elements and in later stages both fibers and vessel elements mature. During this second stage of secondary growth, vascular development in *A.*

thaliana is similar to that of woody species and can be used as a model to address basic questions about wood ontogeny (Chaffey et al., 2002).

Secondary growth resulting in a thicker cambium also occurs in the roots. Transcriptome analysis suggests the involvement of class III HD ZIP and KANDI TFs in the radial patterning during secondary growth of the root (Zhao et al., 2005). More specifically, it has long been known that AtHB8, a class III HD ZIP TF, is necessary for proliferation and differentiation of xylem and is induced by auxin during secondary growth (Baima et al., 2001). The secondary growth of both the hypocotyl and the root vascular tissue is an important area of study for the patterning and differentiation process of xylem vessels in the root.

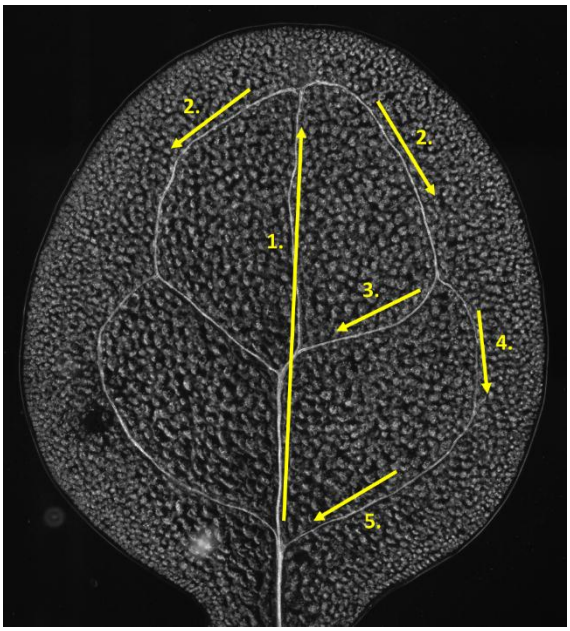


Figure 2. Vascular pattern of the cotyledon. *A. thaliana* wildtype cotyledons were cleared with chloral hydrate and viewed under darkfield microscopy. Wildtype cotyledons first establish the primary vein (1), which develops distally and splits into two secondary veins (2). The secondary vein strands rejoin the primary vein (3) and a subsequent secondary vein develops proximally (4). This vein rejoins the primary vein (5) and a complete vascular pattern is formed.

C. Maturation of vascular cell occurs post-embryonically in flat organs

Most vascularized flat organs, with the exception of petals, are highly photosynthetic. The gas exchange due to photosynthetic activity allows for water loss, which creates an enormous biological need for efficient water transport. *A. thaliana* cotyledons, the "seed leaves", begin their development in early embryonic stages as the two apical foci in the heart stage embryo. The vascular pattern of the cotyledon is established in the embryo, but differentiates into mature vascular tissue post-embryonically. It is relatively simple and lends to easy phenotypic quantification (Fig. 2). The primary vein develops distally from the shoot within the cotyledon. This vein splits into secondary veins which extend proximally rejoining the primary vein. In wildtype, four enclosed aerioles are normally observed. True leaves derive post-embryonically from the shoot apical meristem (SAM), which comprises a multipotent cell population. Here, the leaf founder cells flank the SAM and then grow into leaf primordia and later, to mature leaves. The selection of the leaf founder cells is closely associated with auxin transport and class-I KNOTTED 1-like homeobox (KNOX) genes (Jackson et al., 1994). Leaf subepidermal cells are patterned to become procambial cells which later differentiate to make the vascular bundles. As opposed to cotyledons, both patterning and differentiation of vascular tissue occurs post-embryonically. In maturing leaves, the vascular pattern begins by first establishing a central vein and continuous strands form lateral venation similar to that of the cotyledon. Lower order veins form closed lobes, whereas higher order veins may remain unclosed (Reviewed in Townsley et al., 2012).

II. Procambial Pattern Establishment

A. Auxin Canalization Hypothesis

Procambial pattern establishment is necessary for the development of complete vascular networks. The flow of auxin has long been associated with the recruitment of ground cells that will form the procambium (Nelson, 1998, Sundberg et al., 2000, Scarpella et al., 2006). Sachs (1969) first proposed that cells that undergo a small change that allows them to more effectively import auxin are fated to become veins. The directional, polar flow of auxin establishes veins and the diffusion to the surrounding cells prevents nearby cells from becoming vascular tissue. Since then, mechanisms of controlled transport of auxin along the apical-basal axis have been elucidated. It is known that the influx and efflux of auxin becomes more tightly controlled in cells that are destined to become xylem. Polar auxin transport inhibitors that block this process, result in vascular pattern defects suggesting that polar auxin transport is essential for vascular establishment and bilateral patterning (Ni et al., 1999). Evidence that domains of DR5 expression, a synthetic auxin promoter, narrow as the vascular tissue develops and precede AtHB8 procambial marker expression further support this hypothesis (Scarpella et al., 2006). In the current molecular mechanism, PIN1, a transmembrane auxin efflux carrier protein, localizes to basal end of cells in xylem parenchyma to allow for auxin polarity (Galweiler et al., 1998). PIN1 loss-of-function mutants exhibit abnormalities similar to those observed in plants treated with auxin transport inhibitors (Gelweiler et al., 1998, Petrasek et al., 2006, Wenzel et al., 2008). Similar to the DR5 auxin reporter, broad domains of PIN1 prior to pre-procambial specification narrow, or "canalize," during procambial state acquisition (Scarpella et al., 2006, Wenzel et al. 2007). Polar auxin transport, mediated by the efflux carrier PIN1, is therefore crucial for vascular pattern formation (Wisniewska et al., 2006).

PIN1 must localize and continuously recycle at the basal plasma membrane for proper vascular development (Geldner et al., 2001). PIN1 basal localization depends on endocytosis and

endosomal cycling (Geldner et al., 2001). In plants, endosomes function to recycle and sort proteins associated with the plasma membrane and are thought to be equivalent to the Trans-Golgi Network (TGN) (Lam et al., 2007; Grunewald and Friml, 2010). PIN proteins have been observed to colocalize with FM4-64, an endocytic tracer. This suggests that PIN proteins not only localize to the membrane, but are continuously internalized (Paciorek et al., 2005). Additionally, it has been shown that PIN proteins are trafficked from the recycling endosomes to the plasma membrane. The application of Brefeldin A, a fungal toxin that blocks trafficking from recycling endosomes to the plasma membrane, causes the accumulation of PIN in so-called "brefeldin bodies" (Geldner et al., 2001). The recycling of PIN to and from the plasma membrane is mediated by small G-proteins of the ADP-ribosylation factor (ARF) family (Steinmann et al., 1999; Geldner et al., 2003).

ARF proteins are associated with vesicle trafficking and are activated by GTPase activating proteins (GAPs) or deactivated by exchanging GDP for GTP via guanine nucleotide exchange factors (GEFs) (Reviewed in D'Souza-Schorey and Chavrier, 2006). When ARFs are bound with GTP, they bind tightly to membranes and are active. When GTP is hydrolysed, GDP bound ARFs become soluble and associate weakly with membranes (Donaldson and Jackson, 2000). ARFs and their associated regulators (GAPS and GEFs) have been associated with membrane trafficking in other eukaryotes, but their role in plants has yet to be fully elucidated. They regulate the formation and trafficking of vesicles that move to and from the Golgi, and each family of ARF-GEF proteins is specific to vesicular transport steps (Reviewed in Grunewald and Friml, 2010).

The recycling of PIN1 is regulated by GNOM, an ARF-GEF which is blocked by Brefeldin A (Steinmann et al., 1999, Geldner et al., 2001, Kleine-Vehn et al., 2008). GNOM acts

at the late endosome and recycles PIN to the plasma membrane (Geldner et al., 2003), specifically acting at the basal end of the cell (Kleine-Vehn et al., 2008). Loss-of-function *gnom* mutants exhibit severe embryonic defects because of the disturbance of auxin flow, a key morphogen (Steinmann et al., 1999). Weak *gnom* alleles show disrupted venation that is also associated with improper polar localization of PIN1 during procambial establishment (Koizumi et al., 2000).

Possibly serving in opposition to GNOM, VAN3 is an ARF-GAP that localizes in close proximity to the TGN and functions in endocytosis (Koizumi et al., 2005, Sieburth et al., 2006) . Similar to weak *gnom* alleles, VAN3 loss-of-function mutants exhibit vascular discontinuities including vascular islands in cotyledons, leaves, sepals, and petals (Deyholos et al., 2000, Koizumi et al., 2000, 2005, Sieburth et al., 2006). VAN3 is required for the establishment and maintenance of PIN1 at the plasma membrane and for the recycling transport of PIN1 from the plasma membrane to the endosome (Sieburth et al., 2006). Genetic interactions between VAN3 and GNOM suggest the importance of VAN3 for the polar localization and recycling of PIN1 (Koizumi et al., 2005). Recently, GNOM and VAN3 were shown to colocalize with endocytic membranes that are coated with clathrin, consistent with a role in endocytosis and internalization of plasma membrane proteins such as PIN1 (Naramoto et al., 2010).

C. Phosphoinositide Signaling in Vascular Patterning

Appropriate phosphoinositide (PI) environments stimulate the activity of GAP-bound ARFs (Wong et al., 2005), thus implicating the PI signaling pathways in PIN1 polarization. PIs are phospholipid derivatives of phosphatidylinositol that are preferentially phosphorylated at the 3, 4, or 5 positions on the inositol head group. Some PIs are short-lived and used in response to extracellular stimuli such as PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2. Other PIs are more stable and

necessary for cell growth. These PIs are distributed along the plasma membrane and include PtdIns(3)P, PtdIns(4)P, PtdIns (3,5)P_2, and PtdIns(4,5)P_2 (reviewed in Mayinger et al., 2012). Serving as coordinators of lipid metabolism, PIs play important roles at the membrane-cytosol interface. It is known that the lipid composition on vesicles is crucial for the proper localization and function of certain proteins. For example, they are thought to recruit the ARF family of proteins to the correct physical locale.

Genetic interactions of the ARF-GAP VAN3 with PI signaling genes, such as CVP2, CVL1, and VAB, suggest a role for PIs in PIN1 polarization during vascular patterning (Carland and Nelson, 2009, Naramoto et al., 2009). VAN3 contains a pleckstrin homology domain that binds PtdIns(4)P, directly implicating the PI signaling pathway in vascular patterning (Koizumi et al., 2005). VAN3 localizes to the PtdIns(4)P-enriched trans-Golgi network (TGN) (Preuss et al., 2006), and its localization is likely mediated by VAB, which also binds PtdIns(4)P via a pleckstrin homology domain (Naramoto et al., 2009). The *vab* mutants exhibit similar phenotypes to VAN3, in that PIN1 is localized to the membrane, but not maintained there (Hou et al., 2010). CVP2 and its closest paralogue CVL1 likely produce the PtdIns(4)P ligand for VAN3, as CVP2 encodes for an inositol polyphosphate 5’ phosphatase which hydroplyzes PtdIns(4,5)P_2 to produce PtdIns(4)P (Carland and Nelson, 2004, 2009). Like VAN3 and GNOM, *cvp2* mutants exhibit discontinuous cotyledon vascular patterning (Carland et al., 2002). Interactions of VAN3 with PI signaling genes support a hypothesis where CVP2 and CVL1 produce the PtdIns4P necessary for localization of VAN3 which is mediated by VAB (Carland and Nelson, 2009, Naramoto et al., 2009). Mutants of *5PTAse13* which exhibit similar phenotypes to CVP2 suggest a greater role of polyphosphate 5’ phosphatases in vascular

patterning (Lin et al., 2005). The proliferation of the auxin signal is assisted by these PI pathways, which serve to locate vesicles by means of lipid signaling and metabolism.

D. Nuclear Auxin Response

PIN1 cycling, mediated by ARF-GAPs and ARF-GEFs, allows for PIN1 to locate to the basal end of the cell. There, high coexpression of PIN1 with MP is observed. MP is a auxin responsive transcription factor that is thought to relay auxin signals in the cell (Berleth et al., 1993, Przemeck et al., 1996, Hardtke and Berleth, 1998). MP mutants exhibit defects in generating the procambium in early embryogenesis, suggesting an important role in vascular patterning. It is speculated that PIN1 and MP work together to generate an auxin-specific response. When auxin signals are deployed in actively growing cells, levels of MP rise and PIN1 is polarized. The actively growing regions become auxin sources and pre-existing procambial cells become auxin sinks. This source-sink relationship exemplifies the auxin canalization hypothesis, which rests on the canalized flow of auxin (Wenzel et al., 2007).

In order to self-regulate, auxin sinks and sources must be distinguished. AXR6, an auxin response gene, shows mutant phenotypes similar to that of MP and importantly, fails to generate vascular precursors. This mutant phenotype suggests that signal transduction along many auxin pathways is necessary for vascular formation (Hobbie et al., 2000). Since differential auxin levels are required to identify an auxin sink from an auxin source, MP must be regulated by an auxin responsive gene. BDL forms a complex with MP in response to auxin; BDL is degraded in the presence of a critical amount of auxin, thus releasing MP to activate auxin-source associated responses (Hamann et al., 2002). Such an auxin-regulated feedback loop controls the gene expression necessary to define cells as auxin-sources and auxin-sinks. In this way, canals of auxin flow are created and vascular patterns are established.

Taken together, these genes play a key process in the patterning process of procambial cells which develop into mature xylem cells post-germination. Auxin acts as a morphogen at the patterning stage, causing ground cells to adopt a procambial fate through the canalization of auxin flow. Post-germination, auxin acts once again as a morphogen to induce the differentiation of vascular tissue. AtHB8, a key procambial marker, is regulated by auxin and modulates the differentiation process from procambial to cambial cells (Baima et al., 2000).

III. PATLs, a role for SEC14-related proteins in vascular development.

Sec14-like phosphatidylinositol lipid transfer proteins are eukaryotic proteins that function at the interface between lipid signaling and lipid metabolism (Ghosh and Bankaitis, 2011). Sec14p, the first member of the Sec14 superfamily, was identified in yeast as a protein that couples lipid metabolism with PI signaling events essential for membrane traffic from the trans-Golgi. Since the discovery of Sec14p, the Sec14 domain has been identified in >1500 eukaryotic proteins that function in a diverse array of cellular contexts. Their high degree of sequence conservation, ubiquitous distribution throughout the eukaryotic world and association in humans with a number of diseases and cancers, points to a critical role for Sec14-like proteins in cellular regulation. Sec14 related proteins are involved in vesicle trafficking in the TGN in yeast (Yanagisawa et al., 2002) and are known to function in the phosphoinisitide signaling pathway in other eukaryotic cells (Bankaitis et al., 2009).

The PATLs are a family of Sec14-related proteins of *A. thaliana* thought to be involved in vesicle trafficking during cytokinesis and polarized cell growth. PATL1 binds PtdIns(5)P, PtdIns(4,5)P_2, and PtdIns(3)P (Peterman et al., 2004). Recent work in the Peterman lab suggests that members of the PATL subfamily of SEC14 proteins may play an important role in vascular development. A phylogenetic survey of land plant genomes comparing Sec-14 domain sequences

has revealed that the PATL family first appeared in true vascular plants (Peterman, person communications). Investigations of spatial PATL1 and PATL2 gene expression showed that expression localizes to all cells of the vascular tissue: in cotyledons, leaves, vascular cylinder of the root, sepals, and filaments of the anthers (Fig 3). Additionally microarray data suggests that PATL1 and PATL2 are increasingly expressed during brassinolide-induced tracheary element differentiation (Kubo et al., 2005) coexpressed with RAB3B, a key protein in the tracheary element differentiation process (Levy, 2009), and overexpressed in the vascular cylinder of the root (Brady et al., 2007). These lines of evidence, have led to our hypothesis that PATL1 and PATL2 are intricately involved in vascular development.

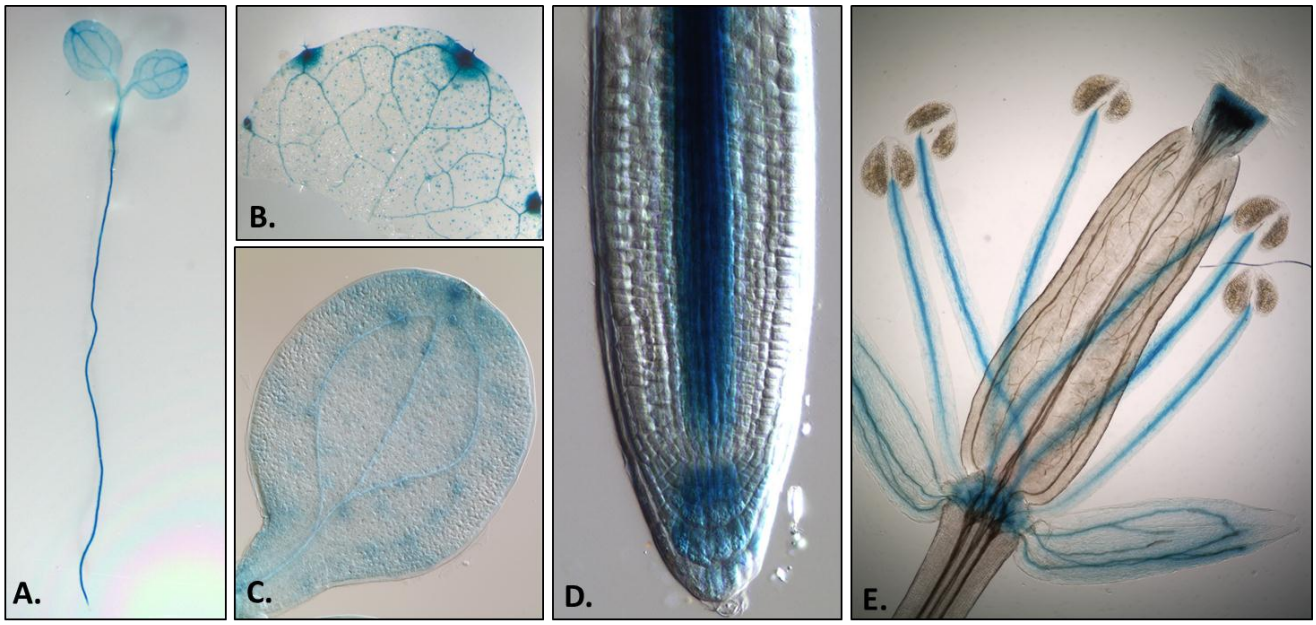


Figure 3. PATL1 is expressed in vascular tissue. Spatial PATL1 expression patterns were observed in PATL1::GUS transgenic plants stained for GUS activity. Blue staining represents sites of PATL1 transcription. Expression is seen in vascular tissue of whole seedling (A), vasculature of the leaf (B) and cotyledon (C), the vascular cylinder of the root (D), and the filaments of the anther and vasculature of the sepal in the flower (E).

In this study, we provide evidence that PATL1 and PATL2 are involved in establishing the procambial pattern in early development. We show that vascular discontinuities increase in *patl1 patl2-1*, double-knockout mutants. This mutant phenotype is similar to, albeit weaker than, that observed in genes necessary for the establishment and maintenance of PIN1 auxin transporter for polar auxin transport. We discovered that PATL1 and PATL2 are expressed in developing procambial cells throughout embryogenesis and in developing leaf primordia. Supporting our hypothesis that PATL1 and PATL2 are involved in PIN1 establishment for polar axin transport, we show that auxin patterns, as monitored by DR5::GFP, are disrupted in *patl1 patl2-1*. Together, these findings suggest that PATL1 and PATL2 are essential for procambial pattern establishment in *A. thaliana,* suggesting an important role for Sec-14-related proteins in regulation of PIs involved in this process.

MATERIALS AND METHODS

I. Plant Material and Growth Conditions

All *Arabiodpsis thaliana* seeds used in this study were obtained from Kaye Peterman, unless otherwise noted. For tissue analysis, unless otherwise noted, sterilized seeds (accomplished by two washes with 1% (v/v) Tween 20, 1 minute incubation in 70% (v/v) EtOH, a sterile water rinse, 5 minute incubation in 30% (v/v) bleach and 6 sterile water rinses) were grown on ½x Murashige and Skoog medium with vitamins, pH 5.5-5.7, (Caisson No. MSP09), 1.0% (w/v) sucrose, and 4.0% (w/v) tissue culture agar. Seeds were vernalized on media at 4°C in the dark for 1-3 days and grown horizontally in a climate-controlled growth chamber (22°C, 16/8-hour photoperiod, ~100 µE s^{-1} m^{-2} light intensity). In cases where plants were grown in soil to maturity, seeds were vernalized in 1% (w/v) agarose at 4°C in the dark and then planted in 75% (v/v) ProMixPGX potting mix (Premier Tech Horticulture) and 25% (v/v) Pro Field Select Conditioner (Pro's Choice) moistened with fertilizer solution (1 g/ liter Peter's 5-5-15 Excel Cal-Mg, 4% (v/v) sulfuric acid). Plants were covered with clear plastic domes and germinated in continuous light at 22°C for about 3 days. After germination, the plants were uncovered and grown in a climate-controlled growth chamber (22°C, 16/8-hour photoperiod, ~100 µE s^{-1} m^{-2} light intensity).

II. Genotyping of PATL1 and PATL2 double-knock out mutants

Transgenic *A. thaliana patl1 patl2* double knock-out mutants were obtained from Kaye Peterman. *patl1 patl2-1* carries SALK_080204 (*patl1*) and SALK_086866 (*patl2-1*) T-DNA insertions and *patl1 patl2-2* carries SALK_080204 (*patl1*) and SALK_009882 (*patl2-1*) T-DNA insertions. *patl1 patl2* double knock-out mutants were genotyped to confirm the presence of the

T-DNA insertions. Polymerase chain reaction (PCR) amplification of mutant DNA with a T-DNA-specific forward primer, BP (LBa1), and a gene-specific reverse primer (Appendix A) results in an ~400 bp fragment. Gene-specific primer pairs were designed to yield a ~900 bp PCR product on wild-type DNA (Appendix A). Mature leaves of either *patl1 patl2-1* or *patl1 patl2-2* were collected and DNA was extracted using REDExtract N-Amp Plant PCR kit following the manufacturer's instructions (Sigma-Aldrich, Cat No. XNAPS-1KT). Extracted DNA was amplified via PCR with appropriate gene specific and T-DNA specific primers (Appendix A) using the REDExtract N-Amp Plant PCR kit and following the manufacturer's instructions. The amplified DNA fragments were analyzed on 1% (w/v) agarose, 1X TAE (40mM Tris, 20mM acetic acid, and 1mM EDTA (pH8)) gels and visualized with SYBR Safe DNA stain (Invitrogen Cat No. G6600) (1:1000). Gels were imaged with UV light using the BioRad ChemiDoc imaging system.

III. Cotyledon Venation Pattern Analysis

For analyis of cotyledon venation patterns, *Arabidopsis* seedlings were grown under sterile conditions for 5 or 9 days. Following growth, seedlings were incubated in depigmentation solution (85.7% (v/v) EtOH, 14.4% (v/v) acetic acid) for 24 hours and washed in 100% (v/v) EtOH, followed by a 70% (v/v) EtOH wash. In some cases, seedlings were stored in 70% (v/v) EtOH. Seedlings were rehydrated with 50% (v/v) EtOH for 30 minutes and cleared in chloral hydrate clearing solution (9:3:1 chloral hydrate: glycerol: water) for at least 12 hours. Cotyledons from each seedling were dissected and mounted in chloral hydrate clearing solution. Slides were sealed and imaged using a Nikon 80i DIC microscope with differential interference contrast (DIC) optics. Images were captured using a QImaging Retiga 2000R camera and NIS Elements AR 3.10 software. Images were assembled and labeled using Adobe Photoshop CS Pro.

Cotyledon venation patterns were assessed using the method of Steynen and Schultz (2003) amended by three additional categories of analysis: frequency of observation of venation gaps, abnormal order of venation development (reverses), and abnormalities in the distal-most branch. Data from 5 day and 9 day seedlings were analyzed separately using a one-way ANOVA test. If means were found to be significant, a Tukey HSD test was performed to assess the variable means. The frequency of gaps, reverses and distal abnormalities were analyzed as a separate dataset and means from 5d and 9d seedlings were combined for one-way ANOVA and Tukey HSD statistical analyses.

IV. Spatial expression patterns of *PATL1* and *PATL2*

Spatial patterns of gene expression were analyzed in transgenic lines carrying *PATL1* or *PATL2* promoter::GUS reporter gene fusions by histochemical staining for β-glucuronidase (GUS) activity Three independent lines were employed for each construct (*PATL1::*GUS lines D1, H1 and K3; *PATL2*::GUS, lines A, B, C). An AtHB8::GUS line (ATCC # CS296) was used as a positive control for GUS activity histochemical staining reactions.

A. Embryonic Tissue

Embryonic tissue was harvested and stained for GUS activity following the protocol outlined in Stangeland and Salehian (2002) with the following modifications. Siliques of mature plants were harvested and slit longitudinally with a scalpel in water. Dissected embryos were moved to a multi-well dish containing approximately 3 mL of X-gluc staining solution (0.5% Triton, 50mM sodium phosphate, 7.2, 2 mM ferrocyanide, 2 mM ferricyanide, 2 mM 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (GOLD Biotechnology). Embryonic tissue was vacuum infiltrated twice for ten minutes and incubated in the dark at 37ºC for 12 hours or until tissue was

sufficiently stained. Stained embryos were then fixed with a 1:1 EtOH: acetic acid solution overnight and cleared with Hoyer's Light without Gum Arabic (1.67 g/mL (w/v) chloral hydrate in water) overnight. Embryos were further dissected on the slide in 10% (v/v) glycerol and mounted in 10% (v/v) glycerol. Stained embryos were analyzed with a Nikon 80i compound microscope using differential interference contrast (DIC) optics. Images were captured using a QImaging Retiga 2000R camera and NIS Elements AR 3.10 software. Images were assembled and labeled using Adobe Photoshop CS Pro.

B. Leaf Primordia

GUS staining of developing leaf primordia was performed according to the procedure of Scarpella et. al. (2004) with the following modfication. Four day old sterile seedlings were removed from medium and permeabilized in 90% (v/v) acetone for one hour at -20°C. Seedlings were washed twice with 50mM sodium phosphate, pH 7.5 and incubated with X-gluc staining solution (0.5% Triton, 50mM sodium phosphate, pH 7.5, 2 mM ferrocyanide, 2 mM ferricyanide, 2 mM 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (GOLD Biotechnology Cat. No. G1281C1)) in the dark at 37ºC for 1.5 hours. Whole seedlings were fixed in 3:1 EtOH: acetic acid for one hour and stored in 70% (v/v) ethanol at 4°C. Seedlings were rehydrated in a series of EtOH washes which included 50% (v/v) and 25% (v/v) washes, followed by a water wash. The leaf primordia were dissected into water and mounted in 8:3:1 chloral hydrate: glycerol: water. Stained leaf primordia were analyzed with a Nikon 80i compound microscope using differential interference contrast (DIC) optics. Images were assembled and labeled using Adobe Photoshop CS Pro.

V. Production of PIN1:GFP, DR5::GFP, and AtHB8::GUS *patl1 patl2* lines

patl1 patl2 double knock-out mutant plants were grown until the first flowers formed from the primary inflorescence. Plants were emasculated using a scalpel to remove the anthers under a dissecting microscope. The stigmas of *patl1 patl2-1* double-knockout flowers were pollinated with anthers of one of the experimental lines (Table 1). Seeds from the crossed plants (T1) were collected and potted in soil; plants were grown to maturity and allowed to self-fertilized. The seed from this T2 generation were screened for homozygosity for all three genes. For the PIN1:GFP and DR5::GFP crosses, plants were grown on sterile media and roots were analyzed for the predicted patterns of GFP expression. The GFP positive seedlings were then genotyped for the *patl1 patl2* T-DNA insertions. Plants carrying all three genes were moved to soil and embryonic expression of the GFP reporter gene was examined using confocal microscopy. In AtHB8::GUS crosses, plants were grown to maturity and leaf tissue was genotyped for the *patl1 patl2* T-DNA insertions. Leaf tissue from confirmed *patl1 patl2* plants was stained for GUS activity. GUS-positive, *patl1 patl2* plants were propagated examined for embryonic AtHB8::GUS expression.

VI. Confocal Imaging of DR5::GFP *patl1 patl2-1* Embryos

Siliques of mature plants homozygous for DR5::GFP in the *patl1 patl2-1* background were harvested in water. Embryos at late cotyledon stage were dissected from the seed coat and mounted in 10% (v/v) glycerol. Slides were viewed using a Leica SP5 confocal microscope with excitation at 488nm.

Table 1. Seed lines crossed into *patl1 patl2*

Seed Line	Description	Obtained From	Cross Performed (♀ x ♂)	Expected Ratio of Homozygosity for all Three Traits in T2
PIN1::PIN1:GFP *patl1*	PIN1 promoter driving expression of PIN1-GFP fusion protein in *patl1*	Kaye Peterman	*patl1 patl2-2* x PIN1::PIN1:GFP *patl1*	1/16
DR5::GFP	DR5 synthetic auxin promoter driving GFP reporter expression in *patl1*	Kaye Peterman	*patl1 patl2-1* x DR5::GFP *patl1*	1/16
AtHB8::GUS	AtHB8 promoter (a procambial cell marker) driving GUS reporter expression	Kaye Peterman	*patl1 patl2-1* x AtHB8::GUS	1/64

RESULTS

patl1 patl2 Double Knock-Out Mutants Exhibit Vascular Defects

To determine if *PATL1* and *PATL2* function in vascular development, *Arabidopsis thaliana* individuals homozygous for T-DNA insertions in *PATL1* and in *PATL2* were crossed to produce a double knock-out of both genes (Fig. 4A). *patl1 patl2-1*, a double knockout line carrying SALK_080204 in PATL1 and SALK_086866 in PATL2 was generated. The presence of these T-DNA insertions in *patl1 patl2-1* was confirmed by amplifying wildtype and T-DNA insertion regions of *PATL1* and *PATL2*. Using gene specific primers (GSP), 900 bp DNA fragments of the wild-type *PATL1* and *PATL2* genes were amplified from wild-type Col DNA as expected (Fig 4B). The presence of the T-DNA insertions in single (*patl1 or patl2-1)* and double (*patl1 patl2-1)* mutants was confirmed by amplifying a region flanked by the T-DNA border primer (BP) and a reverse primer specific for either *PATL1* or *PATL2*. The expected size of the T-DNA insertion fragment is 410+N bp, where N is the distance from the end of the T-DNA insertion and the endogenous gene reverse primer. The predicted 710 kb T-DNA insertion fragment for the *patl1* insertion was observed in the *patl1* single mutant and the *patl1 patl2-1* double mutant. Likewise the predicted 620 kb T-DNA insertion fragment for the *patl2-1* insertion was observed in the *patl2-1* single mutant and the *patl1 patl2-1* double mutant. These results confirm that the *patl1 patl2-1* line contains T-DNA insertions in both the *PATL1* and *PATL2* genes and suggests that these are null alleles. Western blots confirmed that *patl1 patl2-1* does not produce the PATL1 and PATL2 proteins (K. Peterman, personal communication). Similar results were obtained with *patl1 patl2-2*, a second double knockout line with SALK_080204 in PATL1 and SALK_009882 in PATL2 (data not shown).

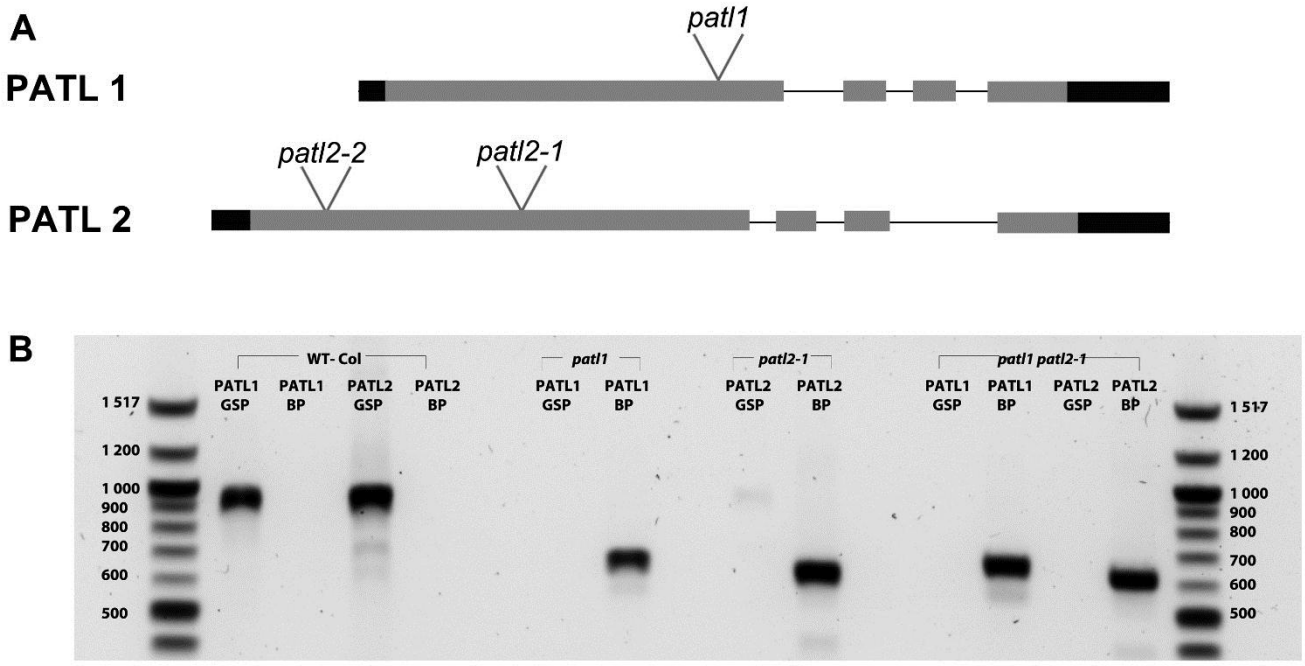


Figure 4. Genotype analysis of *PATL1* and *PATL2* T-DNA insertion mutants. Position of T-DNA insertions in the *PATL1 and PATL2* genes. *patl1* carries SALK_080204, *patl2-1* carries SALK_009882 and *patl2-2* carries SALK_086866(**A**). The presence of T-DNA insertions was verified by PCR using T-DNA (BP) and gene specific (GSP) primers. DNA was separated using gel electrophoresis to visualize the single (*patl1* and *patl2-1*) as well as double (*patl1 patl2-1*) knockouts against a wildtype control (**B**). The expected wildtype fragment for both genes was 900 bp. The expected fragment size for *patl1* was 710 kb and 620 kb for *patl2-1*.

The double knockout lines, *patl1 patl2-1* and *patl1 patl2-2*, were analyzed for defects in cotyledon venation in order to observe whether the vascular pattern was disrupted. Cotyledon venation pattern was chosen for analysis, because it is simple and easily quantifiable. Cotyledons of Col-0 wildtype, *patl1* and *patl2-1* single knockouts, as well as the double knockout lines *patl1 patl2-1* and *patl1 patl2-2* were cleared and scored for venation defects as detailed by Steynen and Schultz (2003). Each cotyledon was scored for the number of free ends, the number of complete aerioles, the number of branch points, and the number of secondary veins (Fig. 5A). The number of free ends was significantly higher in the *patl1 patl2-1* and *patl1 patl2-2* double knockout lines as compared to the wildtype (Fig. 5B). Where the wildtype cotyledons had on average one free end, the mutant cotyledons had on average 1.5 free ends. Also the number of complete aerioles was significantly lower in *patl1 patl2-1* and *patl1 patl2-2* double knockout lines, as compared to the wildtype. The *patl1 patl2-1* and *patl1 patl2-2* double mutants did not differ from wildtype when scored for the number of branch points and the number of secondary veins (Fig. 5B). *patl1* and *patl2* single knockout mutants did not exhibit phenotypes that were statistically different from the wildtype (Fig. 5B) indicating that PATL1 and PATL2 serve redundant or overlapping roles in vascular development.

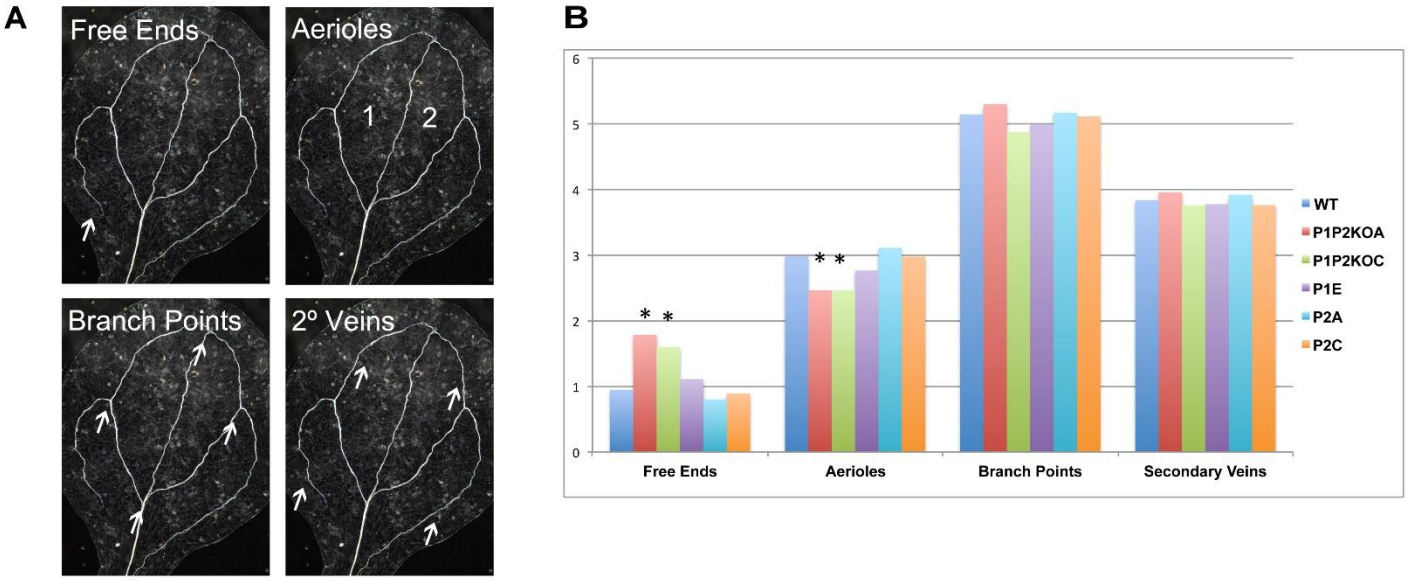


Figure 5. ***patl1 patl2*** **Double Knockout Mutants Exhibit Defects in Vascular Continuity.** The cotyledon vascular pattern was quantified along four parameters that are common to wildtype cotyledons: the number of free ends, complete aerioles, branch points, and secondary veins (**A**). Cotyledon pattern of double (*patl1 patl2-1 and patl1 patl2-2*) and single (*patl1*, *patl2-1*, *patl2-2)* mutants was quantified. Asterisk indicates statistical significance in comparison to the wildtype (Free ends: One-way ANOVA, n=2644, df=5, F ratio= 22.4102, p<0.0001; Aerioles: One-way ANOVA, n=2644, df=5, F ratio= 18.1872, p <0.0001) (**B**).

In order to determine the penetrance of the phenotype, cotyledons were scored for the frequency of vascular defects. Three categories were used: gaps, vein order reversals, and distal defects. Gaps (Fig. 6G, H), or discontinuities in the venation pattern were observed in 28% of the *patl1 patl2-1* cotyledons, which was significantly greater than the frequency observed in wildtype (4%) (Table 2). Vein order reversals, or the formation of secondary veins from the primary vein proximally rather than the secondary vein distally (Fig. 6E), were observed at a frequency of 12% in *patl1 patl2-1*, which was significantly higher than the frequency observed in wildtype (2%) (Table 2). Distal defects, either discontinuities or extra branches at the distal-most region of the cotyledon (Fig. 6 C, D), were observed at a frequency of 13% in *patl1 patl2-1*, which was higher than the frequency observed in wildtype (2%) (Table 2). The frequencies of gaps, reversals, and distal defects in the single knockout lines were not different from those of wildtype (Table 2). *patl1 patl2-2* showed a greater frequency of gaps (18%), but was not significantly different from wildtype in the reversal and distal defect categories (Table 2). We also observed reduced venation patterns (Fig. 6B), whereby the same number of aerioles was observed, but they were significantly smaller. Taken together, *patl1 patl2-1* shows a significant, albeit low, frequency of defective vascular phenotypes.

Table 2. Frequency of Gaps, Reversals, and Distal Defects in *patl1 patl2* Double Knockout Mutants. *patl1 patl2* double knockout mutants were analyzed for the frequency of gaps, reverses, and distal defects alongside wildtype and single knockouts. Asterisk indicates significance from the wildtype.

	Gaps (%)	**Reverses (%)**	**Distal Defects (%)**	n=
Wildtype-Col	4.27	2.27	2.36	230
patl1 patl2-1	28.63*	12.06*	13.62	231
patl1 patl2-2	18.49	5.89	2.48	300
patl1	7.40	3.92	4.30	173
patl2-1	1.49	6.48	2.97	120
patl2-2	5.38	5.73	2.40	258

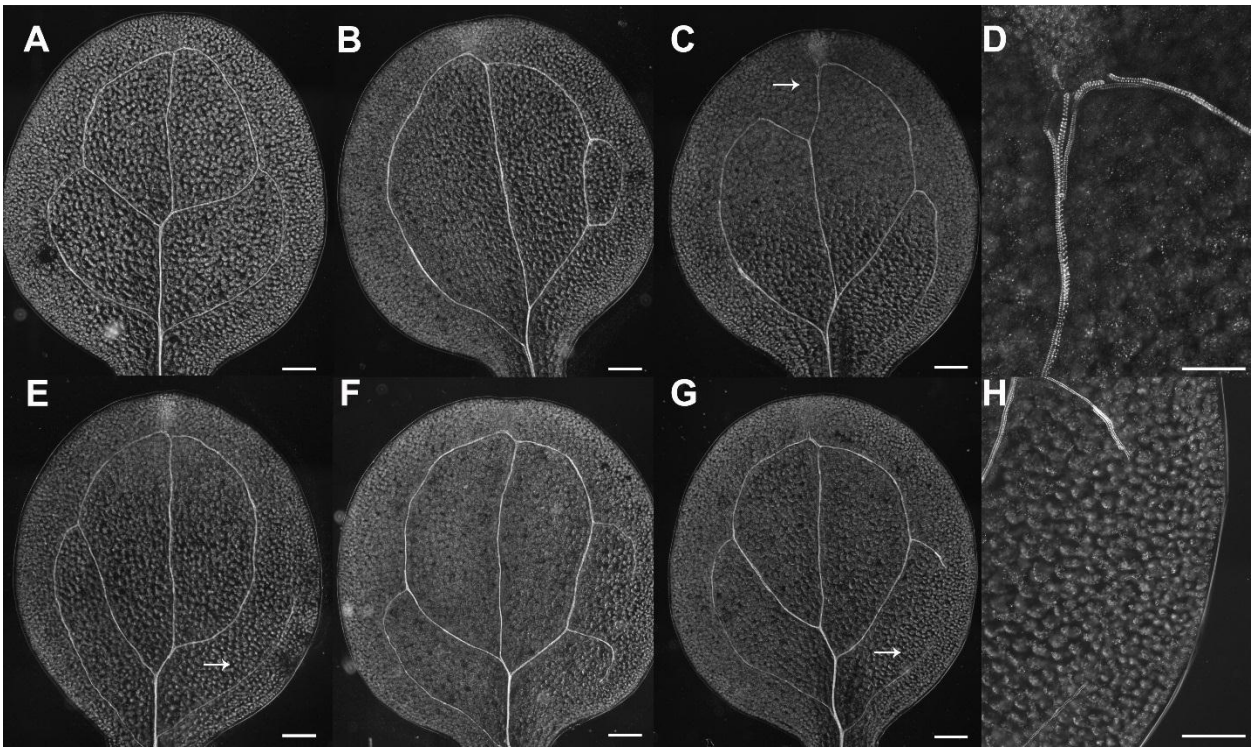


Figure 6. Vascular Patterning Defects in *patl1 patl2* Double Knockout Mutants. The wildtype venation pattern of the cotyledon (**A**) is disrupted in *patl1 patl2* double knockout mutants (**B-H**). Reduced venation (**B**), distal defects (**C**), (higher magnification of distal region (**D**)), reversals in vein pattern (**E**), increased numbers of free ends (**F**), and gaps (**G**), (higher magnification of gap region (**H**)) were observed. Scale bar= 200 um.

PATL1 and PATL2 are expressed in the procambial cells of the developing embryo

The formation of a complete vascular network is the result of a continuous procambium that is established early in embryogenesis. The vascular patterning defects in *patl1 patl2-1* suggest that procambial cells are not established correctly during embryogenesis and predict that PATL1 and PATL2 will be expressed in the preprocambium and/or procambium. Procambium is formed in the developing embryo and is marked by the differentiation of ground cells into elongated files of procambial cells. Procambial cells in the root and cotyledon are arrested in the embryo until germination, when they give rise to cells that differentiate into the xylem and phloem tissues. After germination, vascular development continues in the shoot apical meristem to give rise to vein patterns in the leaves and the vascular differentiation in the cotyledons occurs. We investigated whether *PATL1* and *PATL2* are expressed during the formation of the procambium by observing the expression patterns of PATL1::GUS and PATL2::GUS in developing embryos. PATL1::GUS and PATL2::GUS promoter::reporter constructs allow visualization of *PATL1* and *PATL2* gene expression, as the respective promoter drives the expression of the GUS gene which codes for β-glucuronidase enzyme. When given the substrate, 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (x-gluc), β-glucuronidase cleaves it to produce a blue product. *PATL1* and *PATL2* promoter::reporter fusion lines were analyzed for the expression of the GUS reporter in the developing embryo. AtHB8::GUS, a procambial marker, was used as a control for penetration of x-gluc substrate into the embryo. At the heart stage, the earliest stage examined, PATL1::GUS staining was not observed (Fig. 7A), whereas the AtHB8::GUS control embryos stained according to the previously established pattern (Baima et al., 1995) (Fig. 7E). The expression of PATL1::GUS appeared to be initiated at the late torpedo stage of the embryo when staining was localized to the procambial cylinder of the root and the

procambial precursors in the developing cotyledons (Fig. 7B). AtHB8::GUS expression in the late torpedo stage localized to procambial tissue to a much greater degree than PATL1::GUS (Fig. 7F). At the late cotyledon stage of the embryo, PATL1::GUS expression localized to elongated files of cells, the hallmark of developing procambial cells. (Fig. 7C). This expression pattern was also observed in the AtHB8::GUS procambial marker control (Fig. 7G). Under higher magnification, we observed that PATL1::GUS expression precedes the procambial cell elongation suggesting that PATL1 may direct procambial state acquisition (Fig. 7D). AtHB8::GUS expression localized to the procambial tissue, as well as a region distally, which is not destined to become vascular tissue (Fig. 7H). Though PATL1::GUS expression mirrored that of AtHB8::GUS, we did not see expression of PATL1::GUS at the distal region. PATL2::GUS expression was similar to that of PATL1::GUS in the heart, torpedo, and late cotyledon stage of the developing embryo (Appendix B). Taken together, our data suggest an important role for *PATL1* and *PATL2* in procambial state acquisition and the development of vascular files in the cotyledon.

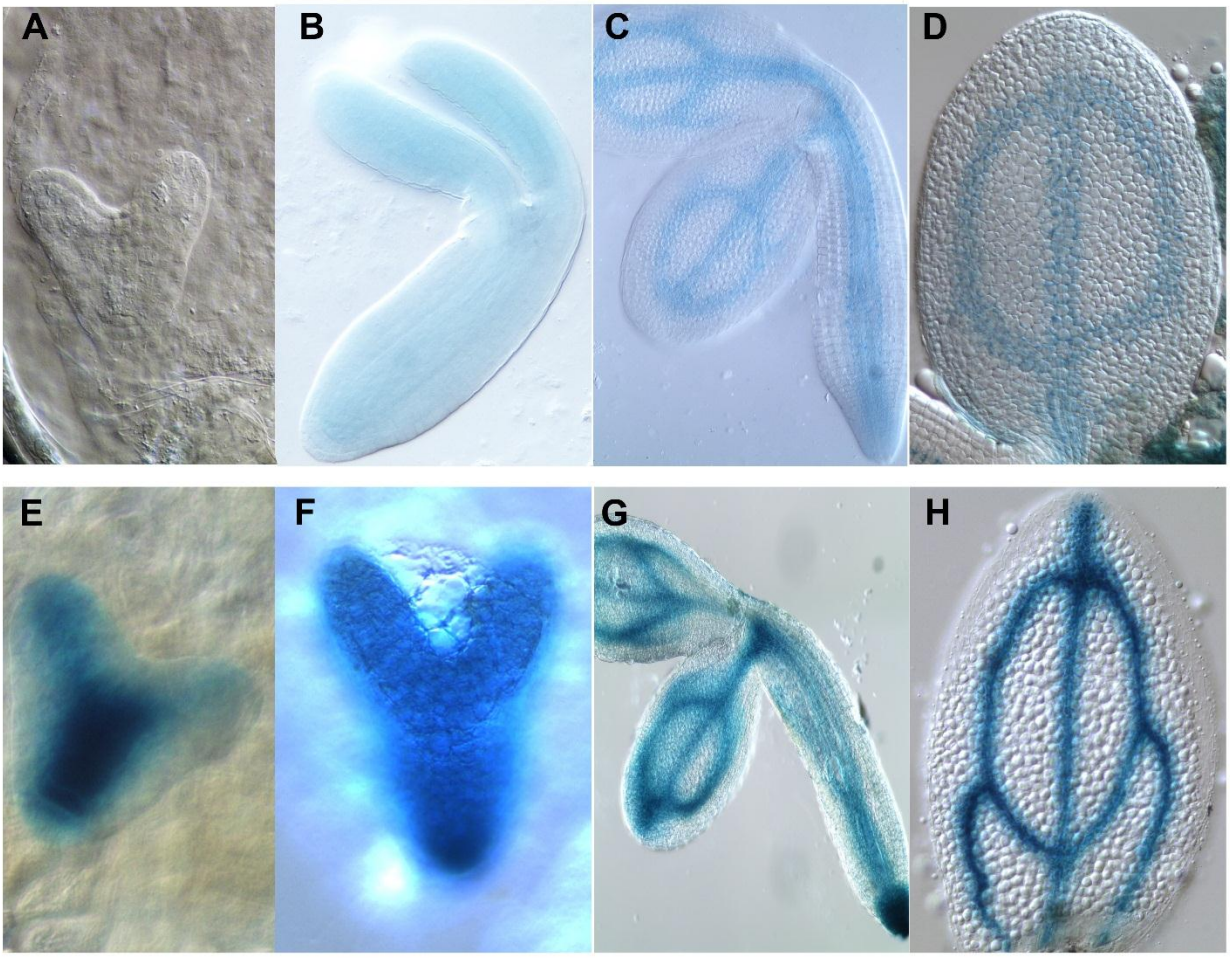


Figure 7. PATL1 is expressed in developing procambium during embryogenesis. Expression of PATL1::GUS promoter::reporter fusions was observed during early embryogenesis in the developing procambial tissue. PATL1::GUS (A-D) was monitored alongside a procambial marker control AtHB8::GUS (E-H). PATL1 and AtHB8 were monitored during the development of the heart stage (A, **E**) the torpedo stage (**B, F**) and in the late cotyledon stage (**C, G**; higher magnification **D**,**H**) of the embryo.

PATL1 and PATL2 are expressed in vascular tissue and vascular precursors after germination

To determine whether *PATL1* and *PATL2* were expressed during vascular development after germination, we investigated the expression patterns of PATL1::GUS and PATL2::GUS in the leaf primordia and mature cotyledons. Mature leaves develop from leaf primordia found between the two mature cotyledons. Here, stem cells of the shoot apical meristem form the developing leaves after germination. Leaf primordia of PATL1::GUS and PATL2::GUS plants were dissected and stained for GUS activity. We observed that PATL1::GUS expression localized in a pattern that predicts the future formation of veins. *PATL1* expression localized to the elongated procambial cells as well as cells just beginning to undergo the process of elongation (Fig. 8A). The expression of PATL1 mirrored the expression of AtHB8::GUS (Fig. 8C), shown at an earlier stage. Interestingly, PATL2::GUS expression did not localize in the same pattern as PATL1::GUS. PATL2 expression appears to precede the formation of mature procambial stands, suggesting localization to preprocambial cells. However a preprocambial pattern that predicts the expected lobed procambial pattern was not observed. PATL2::GUS expression is observed at later stage leaf primordia and it may be that preprocambial expression is present, but does not persist.

Mature cotyledons were cleared and stained for GUS activity in PATL1::GUS and PATL2::GUS lines. PATL1::GUS expression localized with great intensity to the mature vascular strands after just two hours of staining (Fig. 8D). With the exception of stomatal expression, PATL1::GUS was expressed solely in the vascular tissue within the mature cotyledon. PATL2::GUS expression pattern was the same as that of PATL1::GUS, but no stomatal expression was observed (Fig. 8E). The expression of PATL1 and PATL2 were similar

to that of AtHB8::GUS procambial marker, which is known to be expressed in mature vascular tissue after germination (Fig. 8F). These results give evidence that PATL1 is involved in the procambial state acquisition in the leaf primordium, and that PATL2 may serve a different role in this process. Both PATL1 and PATL2 are expressed in mature cotyledon vascular tissue, which echoes the expression seen in the procambial marker control, AtHB8. As such, PATL1 and PATL2 may serve an additional role in differentiation of vascular cells.

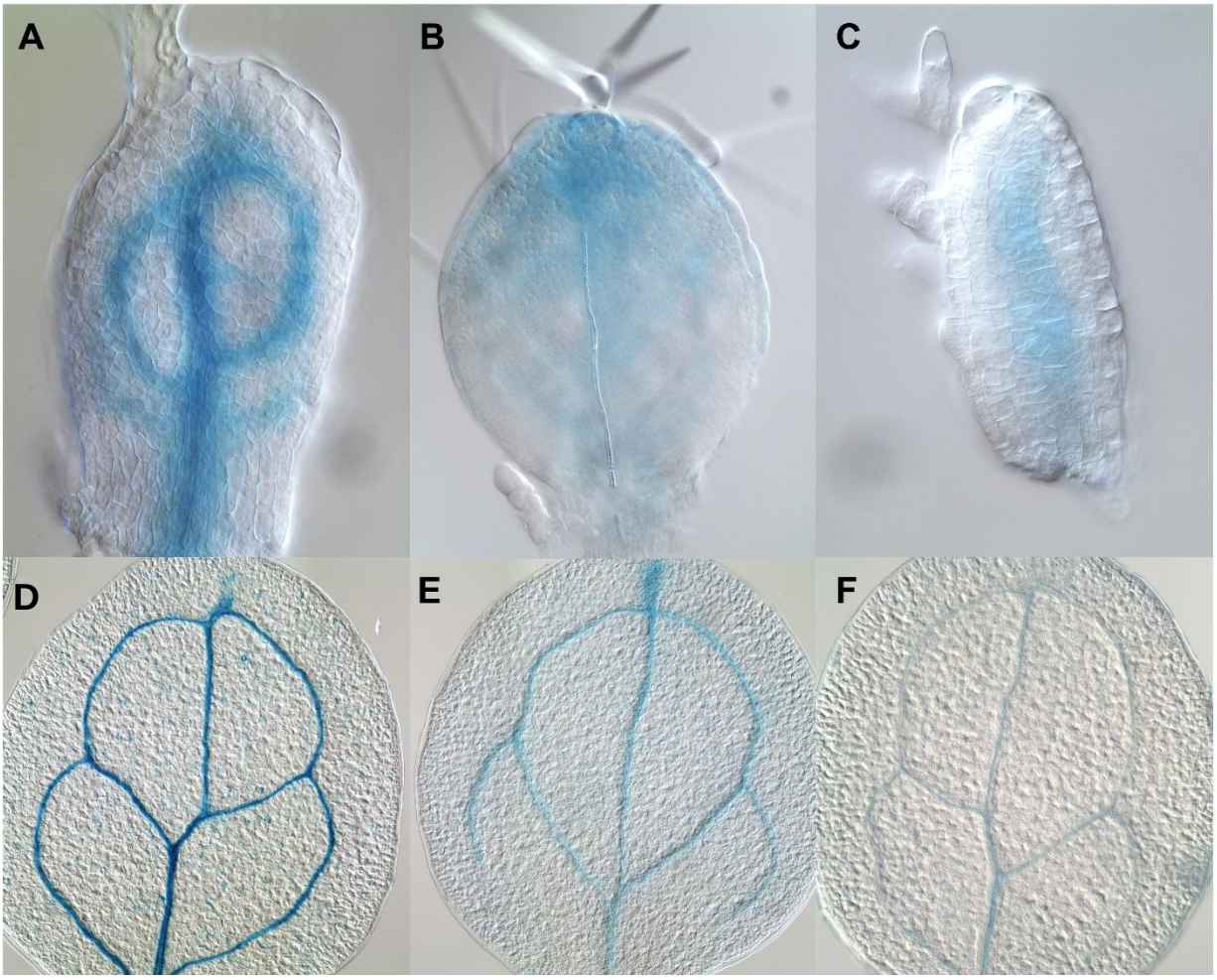


Figure 8. PATL1 and PATL2 are expressed in the developing procambium of leaves and in mature vascular tissue of cotyledons. Leaf primordia (A-C) and mature cotyledons (D-F) were examined for expression of PATL1::GUS (A,D), PATL2::GUS (B,E) and the procambial marker AtHB8::GUS(C,F).

PATL1 and PATL2 Double Knockouts Disrupt Auxin Flow

In order to study the effects of *PATL1* and *PATL2* on the flow of auxin that is crucial for vascular development, we crossed *patl1 patl2* double knockout mutants with plants carrying DR5::GFP or PIN1::PIN1:GFP. DR5 is a synthetic auxin response promoter that has been widely used to evaluate spatial pattens of auxin level and responsiveness (Heisler et al., 2005). We crossed the *patl1 patl2-1* double mutant with a line carrying a construct with the DR5 promoter driving expression of a GFP reporter (DR5::GFP) to determine the effect of PATL1/PATL2 loss of function on auxin canalization. Auxin patterns are canalized during the development of the procambium and the basal localization of PIN1, the auxin efflux carrier protein, is essential for this process (Wisniewska et al., 2006). We crossed the *patl1 patl2-1* double mutant with a line carrying a construct composed of the PIN1 promoter driving expression of a PIN1:GFP fusion protein (PIN1::PIN1:GFP) to allow for examination of PIN1 cellular polar localization in the absence of PATL1/2 function. Products of successful crosses, as determined by PCR genotyping of the PATL1 and PATL2 genes were allowed to self and the *patl1 patl2-1* double mutant homozygote was identified. These lines were also screened using fluorescence for the presence of GFP by observing seedlings under the dissecting microscope for distinctive fluorescence in the root tip. We have identified two lines of DR5::GFP and one line of PIN1-GFP that are homozygous for the *patl1 patl2-1* double mutant.

DR5::GFP expression in the *patl1 patl2-1* background exhibited an increased number of gaps in the pattern (**Fig. 9**). At different stages of procambial establishment, the DR5::GFP expression pattern was interrupted by gaps suggesting that auxin canalization that is required for preprocambial specification was disrupted. Since DR5::GFP reports the location of auxin-induced expression and auxin levels, these results suggest that *PATL1* and *PATL2* are necessary

for correct auxin pattern establishment. Studies are underway to investigate whether DR5::GFP pattern disruption is also observed in leaf primordia and whether PIN1 protein is mislocalized.

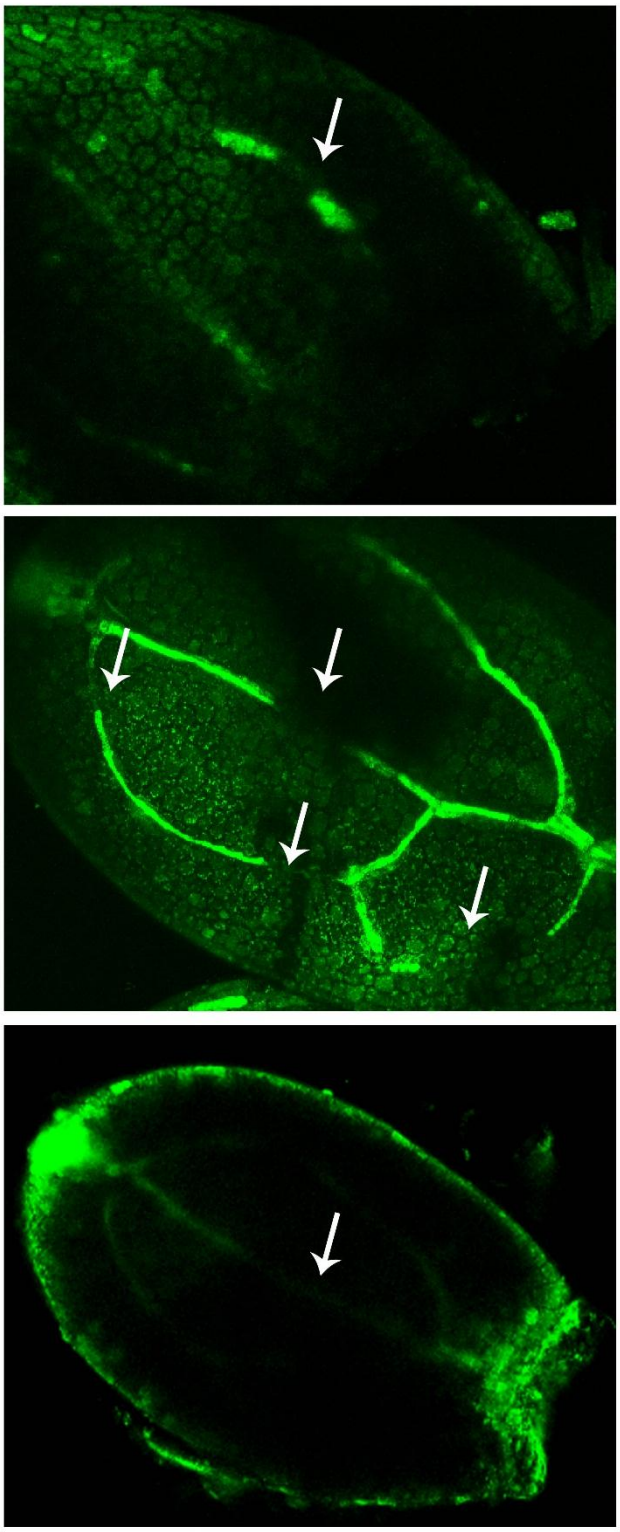


Figure 9. Auxin canalization is disrupted in *patl1 patl2-1* during embryogenesis. Cotyledon stage embryos were dissected and viewed under confocal microscope for GFP fluorescence. DR5::GFP expression was observed in *patl1 patl2-1* lines. Arrows indicate gaps in the pattern.

DISCUSSION

The complex network of veins in leaves and cotyledons is composed of xylem and phloem cells. Establishment of such a pattern begins early in embryonic development when some cells become destined to be preprocambial cells and later procambial cells—multipotent stem cells that will give rise to xylem and phloem. We hypothesized that *PATL1* and *PATL2* may be involved in vascular formation because PATL1::GUS and PATL2::GUS constructs are expressed in the vascular tissue throughout the plant (Levy, 2009). This study has examined the function of *PATL1* and *PATL2* in vascular development and pattern establishment. The results presented here suggest that *PATL1* and *PATL2* play essential and redundant roles in preprocambial pattern establishment and possibly, the maintenance of auxin signals that result in continuous vascular development in leaves and cotyledons.

I. PATL1 and PATL2 are necessary for vascular pattern continuity

Characterization of the vascular pattern of *patl1 patl2-1* cotyledons revealed significant discontinuities. Gaps in the venation pattern were observed in 29% of *patl1 patl2-1* cotyledons. Gaps were observed in secondary venation, and also interestingly, in the primary vein. Indicative of increased vascular discontinuity, we also observed that the number of complete aerioles decreased in *patl1 patl2-1* as compared to the wildtype. Along with an increased number of gaps and a decrease in the number of complete aerioles, we observed a higher frequency of reversals in the vein pattern and distal defects. The reversals of the vein pattern were noted at 12% in *patl1 patl2-1* which was significantly higher than the wildtype. Usually, reversals occurred in the distal secondary veins. Disruptions or discontinuities in the vein pattern were observed at the distal end at a 13% frequency and significantly higher than the wildtype . These disruptions were observed as either unusual aeriole pattern or extra branching at the distal end. Though this category was

broad, defects at the distal end are not reported in the literature of the known vascular patterning mutants. These defects may be explained by the established AtHB8 expression pattern. The AtHB8::GUS promoter::reporter constructs localize to the pre-procambial cells in the embryo and predict vascular strand formation in mature cotyledons; however, strong expression at the distal end of the cotyledon is almost always reported (Koizumi et al., 2000; Kang and Dengler, 2004, Fig. 7H). The localized expression of a procambial marker at the distal end, where veins eventually do not form in mature wildtype cotyledons, may suggest that the signal is diverted proximally during the developmental course. As such, *patl1 patl2-1* mutants may be unable to relocalize the signal at the distal end and therefore exhibit distal defects. Further studies investigating distal pattern formation will be needed to elucidate the role of *PATL1* and *PATL2* in this process.

We observed that *patl1* and *patl2-1* single knockout mutants did not show any a significantly different vascular phenotype from the wildtype. This suggests that *PATL1* and *PATL2* serve redundant functions in vascular patterning, and supports previous phylogenetic analyses that place PATL1 and PATL2 in the same subgroup (Peterman et al., 2006). PATL6, though belonging to a different subgroup (Peterman et al., 2006), may also be implicated in vascular development. Microarray studies of the developing embryo suggest high expression of PATL6 in the preprocambial cells of the globular and heart stage embryo (Xiang et al., 2011). Future studies of vascular defects in *patl1 patl2-1 patl6* will be essential to determine the role of the PATL family in vascular pattern establishment.

The discontinuities observed in the venation of *patl1 patl2-1* cotyledons suggest that *PATL1* and *PATL2* are essential for procambial patterning. The establishment of auxin canals by polar cellular localization of PIN1 auxin efflux proteins patterns ground cells to develop into the

procambium. PIN1 localization to the basal end of the cell is mediated by ARF-GAPs/GEFs through the endomembrane trafficking system. Interestingly, genes associated with the trafficking of PIN1 exhibit a similar phenotype to that observed in *patl1 patl2-1*. An ARF-GAP, VAN3, and its associated protein, VAB, exhibit vascular discontinuities and are known to be essential in the maintenance and recycling of PIN1 at the membrane. Gaps were often observed in *van3,* and strong alleles of *van3* express gap extremes, or vascular islands (Steynen and Shultz, 2003). Interestingly, the primary veins are almost always complete in *van3* mutants, and yet we observed gaps in the primary vein to be common in *patl1 patl2-1* (Steynen and Shultz, 2003; Deyholos et al., 2000; Koizumi et al. 2000). Weaker alleles of *van3* have similar, albeit higher, number of complete aerioles to *patl1 patl2-1* (Carland and Nelson, 2009). In contrast, *vab* expressed gaps that appear of similar size and location in secondary veins to those observed in *patl1 patl2-1* (Steynen and Shultz, 2003). Additionally, reversals are common in the *vab* mutant, occurring in both distal and proximal secondary veins (Steynen and Shultz, 2003).

An ARF-GEF, GNOM is also associated with the trafficking of PIN1 to the membrane and strong *gnom* alleles show dense, randomly ordered tracheids (Mayer et al., 1993); whereas weak alleles of *gnom* had low vascular strand interconnectedness and greater numbers of free ends (Geldner et al., 2003). PIN1 localization is also affected by a 5PTase, CVP2 which cleaves PtdIns(4,5)P_2 into PtdIns(5)P and is associated with the protein CVL1. Both CVP2 and CVL1 are thought to be involved in the recruitment of ARF-GAPs (Carland and Nelson, 2009). Like in *patl1 patl2-1, cvl1* mutants have a low frequency of the gap phenotype; however, *cvp2 cvl1* mutants show a significant increase in gaps and an increase in the vascular island extreme phenotype, indicating the CVP2 and CVL1 act in concert (Carland and Nelson, 2009). Further investigations exploring genetic interactions between *patl1 patl2-1* and either *cvp2, cvl1,* or *vab*

would suggest whether PATL1 and PATL2 also play a role in the ARF-GAP/-GEF and PI-dependent trafficking of PIN1 that is required for establishment of a continuous vascular pattern. It is likely that a cross of *patl1 patl2-1* to *van3* or *gnom* would be lethal, knowing the extreme nature of the venation defect associated with these mutants.

Overall, the present mutant phenotype analysis suggests that PATL1 and PATL2 are necessary for vascular continuity. Complete vascular continuity is compromised in mutants that affect the establishment of PIN1 at the basal end of the cell, a necessary event for cacanalization of auxin flow that leads to procambial cell fate establishment. As such, we speculate that PATL1 and PATL2 are essential for PIN1 localization that leads to procambial patterning.

II. PATL1 and PATL2 expression precedes procambial state acquisition

The vascular pattern in mature cotyledons is established in early embryogenesis. Ground cells elongate and form preprocambial cells, which give rise to procambial cells late in embryogenesis. The loss of cell potency during the development of vascular tissue is associated with the canalization of auxin. Expression of *PATL1* and *PATL2* was observed in developing procambium during embryogenesis and development of leaf primordia. During embryogenesis, we observed *PATL1* expression localized to preprocambial cells of the cotyledon stage embryo. Expression in preprocambial cells was observed in cells that have not yet elongated, but are destined for the procambial state. Similar results were obtained in *PATL2*, further suggesting that *PATL1* and *PATL2* serve redundant functions in cotyledon vascular development.

After germination, pluripotent cells in the shoot apical meristem divide to give rise to the leaf primordia—precursors to mature leaves. For the second time in plant development, procambial cells are established, this time in the leaf primordium which will differentiate into

mature vascular networks. PATL1::GUS expression localized to the preprocambial and procambial cells of the leaf primordia thus predicting where the complex leaf vascular networks will form. PATL1::GUS expression was similar to that seen for the procambial marker AtHB8::GUS. The expression of *PATL1* at the developing hydathodes and trichomes suggests that *PATL1* may be induced by auxin (Aloni et al., 2003). Interestingly, PATL2::GUS expression does not appear to localize to preprocambial and procambial cells with the same specificity as *PATL1*. Instead, broad domains of *PATL2* expression were seen near the differentiating vascular tissue. Though redundancy between *PATL1* and *PATL2* was observed with respect to cotyledon vascular phenotype, these proteins may serve different or partially overlapping functions in leaf vascular development. Future studies investigating mutant phenotypes in leaf vascular pattern may support this conclusion. *PATL1* and *PATL2* are also expressed in mature vascular tissue of the cotyledon. Expression in mature vascular tissue suggests that *PATL1* and *PATL2* are not only involved in the procambial patterning process but also in the differentiation process, supporting public microarray data that noted overexpression of *PATL1* and *PATL2* in a transdifferetiation system of tracheary element cells (Kubo et al., 2005). Additionally, the procambial marker AtHB8 is also expressed in mature vascular tissue of the cotyledon and is thought to function in the differentiation process (Baima et al., 1995).

The spatial expression of *PATL1* and *PATL2* in the developing procambium suggests that *PATL1* and *PATL2* are involved in the procambial pattern establishment. Knowing that localization of PIN1 is essential for procambial state acquisition, it is unsurprising that proteins associated with PIN1 trafficking are also expressed in the developing procambium. Like *PATL1*, the ARF-GEF, *GNOM* is expressed weakly in the procambial tissue at the torpedo stage and localizes to procambial cells in the cotyledon stage. *GNOM* expression precedes vascular

differentiation, an observation we also noted in *PATL1* expression (Geldner et al., 2003). *CVP2* exhibits more specific vascular expression in the torpedo stage embryos than *PATL1* or *GNOM*, but shows similar expression at the cotyledon stage (Carland and Nelson, 2004). At the cotyledon stage, *CVL1*, *CVP2*, and *VAN3* have overlapping expression patterns that localize to the procambial cells (Carland and Nelson, 2009) and replicate the PATL1 expression pattern we observed. *PATL1* expression in the leaf primordium mirrors that of *VAB* (Hou et al., 2010), *CVL1*, *CVP2*, and *VAN3* (Carland and Nelson, 2009). Thus, it is likely that *PATL1* and *PATL2* function in the same pathway as *VAN3, VAB, CVP2,* and *CVL1*.

The present work does not focus on the vascular development in the root, though *PATL1* expression in the root was observed early in embryogenesis. The vascular pattern in the root does not form complex networks as in leaves, but the development of lateral roots is associated with similar hormonal signaling pathways (Reed et al., 1998). *PATL1* expression has been observed in the formation of lateral roots (data not shown) and may be associated with polarized cell growth of root hairs. Investigating the molecular role of *PATL1* in root cells may suggest roles for PATL1 in development of vasculature in leaves and cotyledons.

III. PATL1 and PATL2 are necessary for auxin canalization in the establishment of the procambium

The auxin canalization hypothesis (Sachs, 1969) proposes that plant vascular cell polarity is autoregulated by auxin, which induces its own directionality and rate of transport. It is known that the polar auxin transporter, PIN1 is necessary for auxin efflux from the cell. As the plant vascular system develops, broad domains of auxin and its carrier PIN1 are narrowed, thus establishing procambial cells (Scarpella et al, 2006). The synthetic auxin promoter DR5-driven GFP expression is thought to indicate auxin induced expression and auxin levels. The DR5::GFP

promoter::reporter is expressed in the preprocambial cells of the embryo and is not expressed in elongated procambial cells (Mattson et al., 2003; Carland and Nelson, 2004). We investigated whether DR5::GFP patterns are changed in *patl1 patl2-1* and discovered that auxin-induced expression exhibited significant gaps in the developing vascular pattern of the cotyledon stage embryo. As such, our data suggest that PATL1 and PATL2 play a key role in auxin regulation necessary for preprocambial state acquisition. Since PIN1 cycling at the basal end of the cell is necessary for proper auxin efflux and vascular development, it is plausible that PATL1 and PATL2 regulate vesicle trafficking events necessary for basal maintenance of PIN1. Our data suggest that without PATL1 and PATL2, PIN1 is not properly localized, resulting in gaps in the auxin canals and therefore gaps in the procambium. These data also explain the reduced vascular continuity that we observed in mature cotyledons—if the procambial pattern is established with gaps, the proceeding patterns of vascular differentiation will also contain gaps.

PIN1 is trafficked to and maintained at the membrane by the TGN membrane trafficking pathway. An ARF-GEF, GNOM is known to be essential for PIN1 localization to the membrane and when GNOM is inhibited, PIN1 localizes in small vesicles (Geldner et al., 2003). VAN3, an ARF-GAP, and its associated protein VAB, are PtdIns(4)P binding proteins and are essential for the maintenance of PIN1 at the membrane and recycling it to the endosomes (Koizumi et al., 2005; Sieburth et al., 2006; Hou et al., 2010). In order to stimulate VAN3 and thus PIN1 localization, CVP2 and CVL1 are thought to prepare the appropriate PI environment by cleaving PtdIns(4,5)P_2 to PtdIns(4)P (Carland and Nelson, 2009). PATL1 is known to bind PIs, including the VAN3 ligand PtdIns(4)P (Peterman et al., 2004). Our results show that PATL1 and PATL2 are necessary for complete vascular strand formation and are expressed in the developing procambium. Importantly, the disruption of auxin canals in the developing procambium of *patl1*

patl2 provides evidence that PATL1 and PATL2 are essential for polar auxin transport. As such, it is very likely that PATL1 and PATL2 act in localizing PIN1 to the basal end of the membrane.

It is plausible that PATL1 and/or PATL2 may present the PtdIns(4)P ligand created by CVP2 and/or CVL1 to VAN3 and/or VAB. When bound to PtdIns(4)P VAN3 and/or VAB targets PIN1 maintenance at the membrane, allowing proper vascular development. Future investigations observing PIN1 protein localization will be necessary to confirm this conjecture. PIN1-GFP lines have been crossed into *patl1 patl2-2* and a homozygote has been identified. Confocal imaging of embryonic and leaf primordial expression are underway. Furthermore, investigations of protein interactions of VAN3/VAB with PATL1 and PATL2 will indicate whether PI ligand presentation occurs.

IV. Sec-14 like proteins of A. thaliana are involved in vascular development

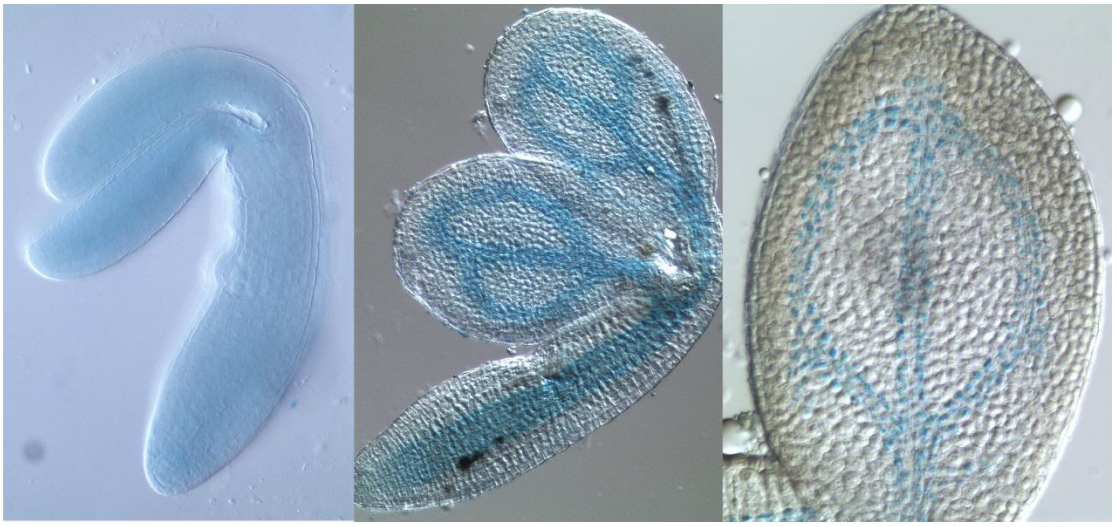
Though the function of Sec14-like proteins has not been elucidated in higher plants, the proposal that PATL1 and PATL2 establish an appropriate lipid environment near the membrane and act as effectors of VAN3 is supported by Sec14 functions in other systems. In yeast, Sec14p is required for vesicle formation in the TGN and acts as an effector to ARF-GAPs. Sec14p creates an appropriate PI environment to stimulate ARF-GAP activity that is required for exit of vesicles from the TGN (Wong et al., 2005; Mousley et al., 2007). In a similar fashion, COW1, an *A. thaliana* Sec14-like protein is required to establish PtdIns4P landmarks that recruit ARF-GAPs that in turn regulate vesicle trafficking during root hair polar growth (Vincent et al., 2005; Yoo et al., 2011). As such, there is a precedent for Sec14-like proteins of *A. thaliana* in the establishment of appropriate lipid environments for vesicle trafficking in the TGN. Our results suggest that Sec14-related proteins modulate PIs that act as effectors of ARF-GAP regulated membrane trafficking, are responsive to auxin, and are involved in vascular development. In

conclusion, the present study proposes the necessity of PATL1 and PATL2 in continuous procambial establishment, suggesting a novel developmental role for Sec14-like proteins in higher plants.

Appendix A. Primer sequences for PATL1 and PATL2 double knock out genotyping

Gene	Insertion Line	Left GSP 5'-3'	Right GSP 5'-3'	Lba1 T-DNA Primer 5'-3'
PATL 1	SALK_080204	TTTGCTTCTTCCTTGGATTTGGA	TCCTGAATTTCGCCGCTAAAGA	TGGTTCACGTAGTGGGCCATCG
PATL 2	SALK_086866	CTCTGACCAAGTCCAGGAGCG	CGTCGCAGAGACCAAGAAGGA	
PATL 2	SALK_009882	CACACATATATTCCCCCATCAAAA	CCTTCCTCCTTAAACGACTCCGA	

Appendix B.



Appendix B. PATL2 is expressed in developing procambium during embryogenesis. Expression of PATL2::GUS promoter::reporter fusions was observed during early embryogenesis in the developing procambial tissue. PATL2::GUS was monitored alongside a procambial marker control AtHB8::GUS (not shown). PATL2 expression was monitored during the development of the torpedo stage (left panel) and in the late cotyledon stage (center; higher magnification right panel) of the embryo.

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